REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	11-28-95	FINAL 6/92 - 11/95

4. TITLE AND SUBTITLE

CHROMOPHORE ATTACHMENT IN THE CYANOBACTERIAL LIGHT_HARVESTING PROTEINS

5. FUNI

6. AUTHOR(S)

LAMONT ANDERSON

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNIVERSITY OF TULSA
600 S. COLLEGE AVE.
TULSA, OK 74104

8. PEI RE IZATION

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. S / ITORING UMBER

11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

Light harvesting in the cyanobacteria is conducted by a complex, self-assembling structure called the phycobilisome, which contains a number of proteins that have covalently linked bilin chromophores (the biliproteins). We have utilized a protein engineering approach to study the structural determinants of chromophore attachment to the biliproteins. As the project developed, we discovered that disruption of the biliprotein subunit structure resulted in severe degradation *in vivo*. We have established that the earliest interactions in the phycobilisome assembly pathway are crucial to the stability of these proteins and must occur accurately and rapidly to avoid degradation. We have examined the role of chromophore attachment in protein stability and the data indicate that covalent attachment of the central bilins in both α and β subunits is needed to stabalize these proteins for assembly. We have used domain exchange experiments to examine chromophore attachment and have found three residues that appear to be required for protein stability and chromophore attachment. We have developed a system for investigating the molecular basis of recognition and docking between biliprotein α and β subunits and have preliminary results from this work.

14. SUBJECT TERMS

cyanobacteria, phycobilisome, chromophore, bilin, proteolysis, phycocyanin, assembly, photosynthesis

15. NUMBER OF PAGES 8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

CHROMOPHORE ATTACHMENT IN THE LIGHT-HARVESTING PROTEINS OF CYANOBACTERIA

FINAL REPORT

Lamont K. Anderson, Ph.D.

November 28, 1995

U. S. Army Research Office

DAAL03-92-G0248

University of Tulsa

The views, opinions, and/or findings contained in this report are those of the author and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

A. Statement of the Problem Studied

The main goal of this project was to employ protein engineering methodology in an attempt to define structural features of the cyanobacterial light-harvesting proteins that are important for covalent attachment of their chromophores *in vivo*. The attachment of bilin chromophores to the biliproteins is an early step in the biosynthesis of the phycobilisome, the cyanobacterial light-harvesting complex. The broad spectrum of visible light that is absorbed by phycobilisomes is a result of spectrally distinct classes of biliproteins. The spectral properties of each biliprotein class are dependent upon protein interactions with the chromophores as well as structural differences in chromophores. All of the major biliprotein classes are similar in structure and sequence, especially in the chromophore binding pockets. As example, the 50 amino acids that comprise the E and F α-helices of the phycoerythrin β subunit (CpeB) and phycocyanin β subunit (CpcB) are identical except for nine residues. Since the E-F helices constitute the chromophore binding site for the β84 bilin in both proteins, the differences between CpeB and CpcB in this region may reflect features that mediate selective chromophore attachment- CpcB has phycocyanobilin attached at Cys84 while CpeB has phycoerythrobilin at the same residue. Protein engineering that exchanged variable E-F residues between CpeB and CpcB can identify possible determinants of selective chromophore attachment.

An examination of chromophore attachment selectivity was the secondary goal of this project. The primary goal was to determine the minimal amount of CpcB structure that was required for chromophore attachment at Cys84 *in vivo*. It was not known if the lyase enzymes that mediate chromophore attachment required an intact, completely folded subunit as substrate as opposed to an unfolded, small fragment of biliprotein. The localization of structural determinants for covalent chromophore attachment to an independent fragment of the biliprotein would help establish the sequence of events in biliprotein biosynthesis and would provide a means of employing chromophore attachment "cassettes" in the development of new smart materials that interact with light energy and that are based upon components derived from biological materials.

In summary, the goals of the project were: (1) establish the minimal amount of protein sequence that would support chromophore attachment at Cys84 on CpcB *in vivo*; (2) examine differences between CpeB and CpcB in the E-F chromophore pocket for possible determinants of selective chromophore attachment. Goal 1 was approached by subtractive protein engineering of CpcB from *Synechocystis* sp. strain 6701. Intact and truncated forms of CpcB were fused to the C-terminal of the maltose binding protein from *E. coli* using the commercial vector, p-Mal (New England Biolabs). The resulting constructs were confirmed as fusions by expression in *E. coli,* and were then moved into a cyanobacterial transformation vector for expression in our host organism, *Synechocystis* sp. strain 6803, strain 4R (a PC-minus mutant). Successful chromophore attachment on truncates of CpcB would be indicated by chromophore-bearing fusion proteins in the transformants. Goal 2 was approached by engineering restriction sites in the *cpeB* and *cpcB* genes that allowed domain exchanges and the construction of various configurations of α-helices between CpeB and CpcB. Expression of a CpcB/CpeB hybrid in 4R would then signal the exchange of regions or residues that are required for specific chromophore attachment on CpcB by the absence of that chromophore (*Synechocystis* sp. strain 6803

does not synthesize PE and cannot attach chromophores to PE subunits, presumably because it lacks the lyase enzymes that mediate selective attachment).

In execution of the experiments, we found that Goal 1 was not achievable due to extreme instability of the fusion products in the cyanobacterium. This observation, coupled with further analysis of the PC-minus mutant (strain 4R) that served as our host organism for transformations (Plank and Anderson (1995) in press, Plant Physiol.; Plank, Toole, and Anderson (1995) in press, J. Bacteriol.), has led to our current research emphasis on subunit interactions, chromophore attachment, and biliprotein stability in the cyanobacterium. We have also further developed our experimental system during this period (Plank and Anderson (1995) in press, J. Bacteriol.) such that we are now exploring the contribution of chromophores to subunit interactions and biliprotein stability (Plank (1995) Ph.D. thesis, Univ. of Tulsa; Plank, Grossman, and Anderson, manuscript in preparation) as well as subunit docking and recognition during the earliest steps of biliprotein self-assembly (Plank (1995) Ph.D. thesis, Univ. of Tulsa). Progress on Goal 2 is near complete. We are at the stage where comparative mutagenesis of only three residues in the E-helix will establish which ones are essential for chromophore attachment to CpcB at Cys84 *in vivo*.

B. Summary of the most important results

Throughout the summary of results, reference to publications derived from this work are designated by **(#)**, indicating the number of the publication from the list of publications in section C.

(1) The cpcBA genes from Synechocystis sp. strain 6803 have been sequenced for the wild-type strain and the 4R (PC-minus) mutant- This work was required in order to explore subunit instability as it relates to protein interactions during phycobilisome assembly and has been published **(2,3)**.

(2) Subunit interactions between α and β are absolutely required for biliprotein stability in vivo- The instability of the maltose binding protein/CpcB fusions may result from a phycobilisome assembly system that rapidly degrades biliprotein subunits that cannot bind to their cognate assembly partner. Analysis of interposon and truncation mutations in both the *cpcB* and *cpcA* genes of *Synechocystis* sp. strain 6803 shows that disruption of the structure of one subunit leads to the *complete absence* of that subunit as well as its assembly partner **(3)**. That subunit instability is a product of proteolytic degradation was indicated by the rescue of intact CpcA from 4R (a CpcB truncation mutant) by heterologous assembly with CpcB from *Synechocystis* sp. strain 6701 **(4)**. These results suggest that the interaction of α and β subunits occurs immediately upon translation in order to confer protection against proteolysis.

(2) Chromophore attachment is required for biliprotein stability and may stabalize the subunit interface, thereby promoting rapid αβ interactions that protect against proteolysis- [Part of this work, primarily mutant construction, was completed

before the grant period. The early data were not easy to interpret until completion of work in this project **(3)** and additional experiments were then performed.] A series of chromophore deletion mutations were constructed in the *cpcBA* genes of *Synechocystis* sp. strain 6701. These constructs were then expressed in the 4R strain of *Synechocystis* sp. strain 6803 and the resulting transformants were analyzed for relative PC content by reversed-phase HPLC and this value was used to indicate the effect that specific chromophore deletion has on biliprotein stability. Single bilin deletion at the Cys84 site in the α or β subunits reduces PC stability by 86% and 78%, respectively. Loss of the peripheral bilin at Cys155 on β causes a 46% decrease in stability. Deletion of the Cys155 bilin on β and the Cys84 bilin of α decreases PC by 91%. The loss of both bilins from β or both Cys84 bilins (β and α) results in complete loss of biliprotein. The loss of each individual bilin has a reproducible effect on biliprotein stability that appears to additive. Decrease in stability may be due to exposure of new proteolysis sites, or subunit interactions may be attenuated by the absence of bilins, causing the some exposure of separate subunits to the cellular environment. As was shown earlier **(3)**, subunits that are not bound to their partners are rapidly degraded.

It is important that attachment of a single bilin at the Cys84 site on α or β confers some protection for both subunits against proteolysis, yet the absence of both of these central bilins causes complete loss of the biliprotein. This observation suggests that bilin attachment at Cys84 stabalizes a subunit's structure such that it can dock with a chromophore-deleted partner, thereby protecting both subunits from complete proteolysis. While the Cys84 bilin is distal to the subunit interface, the turn between α-helices E and F of the β subunit contains a class conserved hydrophobic residue (Phe100 in PC, Leu100 in PE) that packs into a hydrophobic cluster of amino acids on the α subunit. This provides a possible link between Cys84 bilin attachment and the availability of a subunit for binding to its partner- bilin attachment could promote a conformation at the interface that improves α/β interactions. These data are consistent with a model of early events in biliprotein biosynthesis where rapid chromophore attachment at Cys84 occurs before interactions between α and β and the formation of the biliprotein monomer **(5)**. This work is finished and the manuscript is currently in preparation (Toole, Grossman, and Anderson) and will be submitted by January 31, 1996.

(3) Domain exchange experiments between CpcB and CpeB have identified three class-conserved residues in the E helix that are required for protein stability and/or chromophore attachment in CpcB- We constructed a series of restriction sites in the *cpeB* and *cpcB* genes that allowed the exchange of specific combinations of α-helices between the β subunits of PC and PE. One set of domain exchange experiments focused upon the E-F helices that comprise the binding pocket for the central chromophore in the β subunit. Two hybrids were constructed: (I) *XYAB-**EF**-GH* and (II) *XYAB-**E**-FGH* (bold letters indicate α-helices from the PE β subunit, the other letters represents PC β helices). Expression of these hybrid proteins in the *cpc* operon context in the 4R strain demonstrated that both hybrids were completely unstable. Examination of CpcB and CpcE sequences showed that hybrid II differed from wild-type CpcB at only 6 residues in the ***E*** helix. Three of the differences are not conserved between PE and PC and are acceptable

variations within the spectrum of known biliprotein sequences (12 for PE, 18 for PC). The other three residue differences are conserved variations between CpeB and CpcB. Two of the sites (Cys73 and Ala86 in PE, Ala73 and Met86 in PC) have side chains that are within 4 angstroms of the Cys84 bilin attachment residue and are excellent candidates for selectivity determinants in chromophore attachment. The third site is Leu100(PE)/Phe100(PC), which is part of the hydrophobic subunit interface and was discussed earlier in relation to chromophore-dependent protein stability. This explains why hybrid II shows complete instability of PC as opposed to the partial instability that is observed in the chromophore deletion mutation at Cys84 of CpcB (see last section). We are currently exchanging each of these three residues in the ***E*** helix to their PC analogs, individually and in concert. This work should be complete in the first quarter of 1996.

(4) Domain exchange, site mutagenesis, and random mutagenesis of residues at the αβ interface provide a means of examining the molecular basis of recognition between biliprotein subunits during phycobilisome assembly- Inspection of the sequence database (12 PE sequences, 18 PC sequences) shows that the α/β subunit interface is remarkably conserved between PE and PC. Given that the penalty for not binding to an assembly partner is immediate degradation (see section 2), it is of interest how the subunits accurately and rapidly assemble with their proper partners in a cellular milieu that contains high concentrations of structurally similar, but inappropriate, partners. In short, if the PE α subunits are very similar to the PC α, what subtle structural features prevent cross-assembly (PE α with PC β)? The sequence database clearly defines a set of interface residues that are conserved within a biliprotein class. One group of polar residues (2 on α, 2 on β) constitutes a cluster located on the periphery of the interface and shows class-specific charge composition, including a defined salt-bridge between the α and β of PC. The other residues (2 on β, 4 on α), are part of the hydrophobic interface and includes the Phe100/Leu100 residue at the turn between helices E and F.

We are using the exquisite sensitivity of biliprotein subunits to structural disruption as an *in vivo* screen for mutations that affect subunit binding. In principle, mutations at the subunit interface that block or attenuate docking and recognition will manifest themselves in a decreased biliprotein content relative to the wild-type proteins. We have already established that site mutations in the charge cluster in PC, including the introduction of opposing charges at the salt bridge, result in PC levels that range from 0% to 80% of the control **(5)**. The importance of Phe100 to αβ interactions in PC was demonstrated by changing this residue to Gly, which completely abolished PC content in the transformants **(5)**. We have also substituted PE analogs at some of the conserved sites in the PC interface and have observed variable PC stability. We have not exhausted the PE-PC residue exchange possibilities, but are currently focusing on sytematic mutation at the salt bridge and at Phe100 in order to elucidate the importance of these sites for subunit docking and binding.

We have also employed domain exchange with random mutagenesis to study the subunit interface. We constructed the hybrid protein *XYAB-EFGH* where the primary residues at the subunit interface are derived from the PE β subunit (helices ***XYAB***) and all chromophore attachment sites are derived from the PC β subunit (helices *EFGH)*. Expression of this construct with CpcA in the 4R host results in complete absence of any

PC subunits, indicating that the ***XYAB*** domain of the hybrid cannot bind with the CpcA. Random mutagenesis of the ***XYAB*** domain can identify changes that allow some rescue of PC subunits by assembly. A plasmid containing the DNA encoding ***XYAB*** was passaged through an *E. coli* strain that was grown in the presence of nitrosoguanidine as a mutagen. The ***XYAB*** region was then excised and placed into the *cpc* operon in a cyanobacterial transformation vector, creating a plasmid library of mutated β subunit binding domains. the 4R strains was then transformed with the mutagenized plasmid library and clones that displayed a darker blue-green pigmentation (suggesting rescue of PC subunits) were visually selected. In our first pass at this method, we have isolated one clone that shows definite rescue by the presence of low amounts of PC subunits and the DNA from this clone was recently amplified for sequence analysis. This approach should provide us with information regarding residues on the PE β subunit that prevent it from cross-assembling with the PC α subunit. Systematic application of this method, in combination with site mutagenesis, will allow definition of molecular recognition features in the biliprotein subunits.

C. List of publications

(1) Anderson, L., Plank, T., Toole, C., and Cai, J. (1995) Subunit interactions, chromophore attachment, and proteolysis in the assembly of cyanobacterial light-harvesting proteins. Prot. Engineer., Short Reports, 6:17.

(2) Plank, T., and Anderson, L. K. Cloning and sequence analysis of the genes encoding CpcB and CpcA from *Synechocystis* sp. PCC 6803. (in press) Plant Physiology.

(3) Plank, T., Toole, C., and Anderson, L. K. - (1995) Subunit interactions and protein stability in the cyanobacterial light harvesting proteins, in press, Vol. 177, no. 23, *J. Bacteriol.*

(4) Plank, T., and Anderson, L. K. - (1995) Heterologous assembly and rescue of stranded phycocyanin subunits by expression of a foreign *cpcBA* operon in *Synechocystis* sp. 6803, in press, Vol. 177, no. 23, *J. Bacteriol.*

(5) Plank, T. (1995) Early events in the biosynthesis and assembly pathway of cyanobacterial light-harvesting proteins. Ph.D. thesis, University of Tulsa, Tulsa, OK.

D. Participating scientific personnel

Lamont Anderson, Ph.D.	Principle Investigator
Tracey Plank	Ph.D. student; **Ph.D. conferred Aug.28, 1995**
Colleen Toole	Ph.D. student, 3rd year
Jianfeng Cai	Ph.D. student, left program- Sept. 1995
Susan Hettenbach	Research Technician
Tim Tesmer	Undergraduate, now in Ph.D program at Michigan State